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Media Optimization by Central Composite Design Based Response Surface Methodology of Crude α-L-Arabinofuranosidase from *Penicillium* Species

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ABSTRACT:
The aim of the present study was focused on the media optimization for the production of crude α-L-arabinofuranosidase through submerged fermentation technology from soil fungal isolates. The soil sample collected from the foot hills of Anaikatti, Western Ghats, Coimbatore district was screened for the presence of α-L-Arabinofuranosidase producing organisms. The pure colony isolated through serial dilution technique was identified as *Penicillium* species and the crude enzyme activity was found to be 0.004 U/ml. The Plackett-Burman Design (PBD) screening has proved that the variables like agitation rate (150 rpm), orange peel (3.5 %) and $CaCl_2$ (1 g/l) were found to have significant effect in the production of α-L-arabinofuranosidase (0.045 U/ml). The central composite design based response surface methodology (RSM) analysis has confirmed that a combination of high concentration of orange peel (2.5%), low agitation rate (132.95 rpm) and high concentration of $CaCl_2$ (0.75 g/l) yielded a maximum enzyme activity of 0.052 U/ml.

Keywords: α-L-arabinofuranosidase, *Penicillium* species, Plackett-Burman design, response surface methodology

INTRODUCTION

Agro-industrial wastes and byproducts are renewable form of resources generated round the year all over the world. Wheat and rice bran, sugar cane bagasse, corn cobs, citrus and mango peel etc. are the important industrial food wastes. Industries where such wastes and byproducts produced are making strict efforts for their proper disposal. These waste/byproducts, if properly utilized, can widen the economic growth of a country. One of the significant applications of agro-industrial wastes is the biotechnological production of lignocellulolytic enzymes such as cellulases, xylanases, arabinofuranosidases and pectinases can be utilized in the processing of different foods that is not only advantageous quantitatively but qualitatively as well [1]. Majority of the above mentioned industrial enzymes are produced through large-scale submerged fermentation by using agro-industrial wastes as substrate that can act as good sources of carbon and nitrogen [2].

Plant cell walls, the major reservoir of fixed carbon in nature, have three major polymeric constituents: cellulose, hemicellulose and pectin. Various agricultural residues such as corn fiber, corn stover, wheat straw, rice straw, and sugarcane bagasse, contain about 20-40% hemicellulose, the second most abundant polysaccharide in nature. Hemicelluloses are heterogeneous low molecular weight polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids. Unlike cellulose, hemicelluloses are not chemically homogeneous [3] and L-arabinosyl residues are widely distributed as arabinan, arabinoxylan, gum Arabic, arabinogalactan, etc., [4]. Arabinose containing xylans are found mainly as the secondary wall components in gymnosperms and monocotyledonous plants. It consists of a backbone with β-1,4-linked xylopyranose and side chains of α,L-arabinofuranoside, an acetyl group, and/or 4-O-methyl glucuronic acid at the C-2 and C-3 positions of the xylose units [5]. Due to the complexity of xylan structure, its complete degradation requires the cooperative action of several enzymes which include xylanase, β-xylosidase, α-L-Arabinofuranosidase (AFase), acetyl esterase (AE) and α-glucuronidase.

α-L-Arabinofuranosidases (α-L-AFases; α-L-arabinofuranoside arabinofuranohydrolase; EC 3.2.1.55) are exo-type enzymes that catalyze the hydrolysis of non-reducing terminal α-L-arabinofuranosidic linkages present in arabinoxylan, L-arabinan, and other L-arabinose containing polysaccharides [6]. The α-L-AFases have been purified from several bacteria, fungi and plants [7, 8, 9]. They form a part of the array of glycoside hydrolases required for the complete degradation of arabinose containing polysaccharides. The action of these enzymes accelerates the hydrolysis of the glycosidic bonds by more than 10^{17} fold, making them one of the most efficient catalysts known. The α-L-AFases specifically catalyze the hydrolysis of terminal non-reducing-α-L-1,2-, α-L-1,3-, and α-L-1,5-arabinofuranosyl residues from different oligosaccharides and polysaccharides [10]. Whereas, the nature of a glycone sugar can influence the catalytic activity of other arabinose releasing enzymes, the α-L-AFases do not distinguish between the saccharides link to the arabinofuranosyl moiety and thus exhibit wide substrate specificity [9].

Recently, α-L-arabinofuranosidases have received much attention because of their practical applications in various biotechnological processes, such as,

clarification of fruits and vegetable juices, the efficient conversion of hemicellulosic biomass to simple fermentable sugars for subsequent production of fuels and chemicals, delignification of pulp, efficient utilization of plant materials for animal feeds, and hydrolysis of grape monoterpenyl glycoside during wine fermentation [11]. Hence, it is necessary to investigate the variables that affect the production of α-L-arabinofuranosidase through the statistical method of experimental designs like Plackett-Burman design and response surface methodology. Generally these kinds of experimental designs help to identify the optimal conditions, the factors that most influence the results, and those that do not, as well as the existence of interactions and synergies between factors [12].

Eventhough α-L-arabinofuranosidase have been isolated and characterized from many different species, there are lot of scientific documentations to be reported yet. In this concern, our present work has been focused on the media optimization for the production of crude α-L-arabinofuranosidase through submerged fermentation technology from the soil fungal isolates.

MATERIALS AND METHODS

Materials

p-Nitrophenyl-α-L-Arabinofuranoside (PNPA) was obtained from Sigma Chemical Co., (USA). All other chemicals used in the experiments were of analytical grade.

Sample collection and screening for fungal isolates

The soil sample was collected from the foothills of Anaikatti, Western Ghats, Coimbatore, India. About 1g of the soil sample was weighed and dissolved in 100 ml of distilled water (10^{-1}). An aliquot of 1ml was transferred to 9 ml of distilled water to make dilution of 10^{-2}. This was followed till 10^{-6} dilution. Then from each dilutions about 0.1ml of the sample was transferred to the Potato dextrose agar plates using spread plate technique. Potato dextrose agar medium was prepared by weighing 3.9g dissolved in 100 ml of distilled water and sterilized in autoclave for 15 mins at 120°C. The media was then plated evenly in 25 ml petri dish and allowed for solidification. After 72 hrs of incubation the growth of fungal colonies in all the dilutions were observed. Colonies from10^{-4} and 10^{-5} dilution were chosen for pure culture isolation. The genus identification was done using the common fungal stain Lactophenol cotton blue (1 ml of stain mixed with one loopful of colonies, dried and viewed under Reflected light Fluorescence microscope. From the isolated fungal species about one loop full of colonies (10^3cells) were transferred to 100ml Potato Dextrose broth (2.4g in 100ml distilled water and sterilized) and kept under shaking condition (37°C) for one week. The culture filtrate was then centrifuged at 10,000 rpm for 10 mins and the collected supernatant was used for enzymatic studies.

Enzyme assay

A Slightly modified spectrophotometric method described in [13] was adopted to determine the activity of crude α-L-arabinofuranosidase. About 0.2ml of 1mM p-nitrophenyl α-L-arabinofurnoside (PNPA), 0.1ml of enzyme extract and 0.1ml of 50mM citrate buffer is added and incubated at 50°C for about 30 mins. The reaction was stopped by adding 0.5ml of sodium bicarbonate. The liberated p-nitrophenol (yellow colour) was measured spectrophotometrically at 410nm. One unit of the enzyme activity was defined as the amount of enzyme that liberated 1µmol of p-nitrophenol per minute under the assay conditions.

Plackett-Burman experimental design

The significant variables affecting the production of AFase were identified using a 2-factorial system i.e., Plackett-Burman design (PBD) [14]. The effect of individual parameters on enzyme production was calculated by the following equations:

$$E_o = (\Sigma M_+ - \Sigma M_-) / N \quad \text{---------- (Eqn.1)}$$

$$E = \beta_1 + \Sigma \beta_2 + \Sigma \beta_3 + \beta_{123} \quad \text{---------- (Eqn.2)}$$

In Eqn. 1, E_o is the effect of first parameter under study while M+ and M− are responses of enzyme production by the fungal strain. N is the total number of optimizations. In Eqn. 2, E is the significant parameter, β_1 is the linear coefficient, β_2 the quadratic coefficient while β_3 is the interaction coefficient among significant process parameters [15]. The variables selected for the present study includes, pH (4 and 7), orange peel (1.5% and 3.5%), yeast extract (0.1 g/l) and 1 g/l)), agitation rate (150 and 200 rpm), calcium chloride (0.5 g/l and 1.0g/l)), magnesium sulphate (0.5 g/l and 1.0 g/l)) and inoculum size (10^5 and 10^8 spores/loop). The experimental runs obtained were depicted in Table 1.

Response surface methodology (RSM)

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analysing the significance or the influence where the independent variables have on the dependent variable or response. RSM explores the relationships between several explanatory variables and one or more response variables. It was first introduced by G. E. P. Box and K. B. Wilson in 1951 [16]. There are several choices for second-order designs in the RSM. One of the most popular methods is the central composite design (CCD). The effects of agitation rate 150 – 200 rpm), orange peel (1.5% - 3.5%) and $CaCl_2$ (0.5 g/l – 1.0 g/l) on the production of α-L-arabinofuranosidase were studied using a full factorial CCD with 8 factorial cube points, 6 axial points and 6 system recommended centre points (Table 2).

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Table 1: Real values used in Plackett-Burman design (2^7)

Exp.Run	*pH*	*Inoculam size (spores/ loop)*	*Orange peel (%)*	*Yeast extract (g/l)*	*Agitation rate (rpm)*	*$CaCl_2$ (g/l)*	*$MgSO_4$ (g/l)*
1	4	10^8	3.5	1.0	150	1.0	1.0
2	4	10^5	1.5	1.0	200	1.0	0.5
3	7	10^8	3.5	0.1	200	1.0	0.5
4	4	10^8	1.5	0.1	150	1.0	1.0
5	4	10^5	3.5	1.0	200	0.5	1.0
6	7	10^5	3.5	1.0	150	1.0	0.5
7	4	10^8	3.5	0.1	200	0.5	0.5
8	7	10^8	1.5	1.0	200	0.5	1.0
9	7	10^8	1.5	1.0	150	0.5	0.5
10	7	10^5	3.5	0.1	150	0.5	1.0
11	4	10^5	1.5	0.1	150	0.5	0.5
12	7	10^5	1.5	0.1	200	1.0	1.0

Table 2: Real values adopted in full factorial central composite design (CCD)

Exp.Run	*Orange peel (%)*	*Agitation rate (rpm)*	*$CaCl_2$ (g/l)*
1	2.5	132.96	0.75
2	2.5	175	0.75
3	1.5	150	0.5
4	1.5	200	1.0
5	2.5	175	0.75
6	2.5	175	1.17
7	2.5	175	0.75
8	3.5	200	1.0
9	3.5	150	0.5
10	3.5	150	1.0
11	2.5	175	0.33
12	2.5	217.05	0.75
13	2.5	175	0.75
14	4.18	175	0.75
15	2.5	175	0.75
16	1.5	150	1.0
17	3.5	200	0.5
18	0.82	175	0.75
19	1.5	200	0.5
20	2.5	175	0.75

Table 3: Plackett – Burman design (PBD) experimental results of real values

Exp.Run	*pH*	*Inoculam size (spores/ loop)*	*Orange peel (%)*	*Yeast extract (g/l)*	*Agitation rate (rpm)*	*$CaCl_2$ (g/l)*	*$MgSO_4$ (g/l)*	*Enzyme activity (U/ml)*
1	4	10^8	3.5	1.0	150	1.0	1.0	0.043
2	4	10^5	1.5	1.0	200	1.0	0.5	0.014
3	7	10^8	3.5	0.1	200	1.0	0.5	0.029
4	4	10^8	1.5	0.1	150	1.0	1.0	0.036
5	4	10^5	3.5	1.0	200	0.5	1.0	0.019
6	7	10^5	3.5	1.0	150	1.0	0.5	0.055
7	4	10^8	3.5	0.1	200	0.5	0.5	0.019
8	7	10^8	1.5	1.0	200	0.5	1.0	0.011
9	7	10^8	1.5	1.0	150	0.5	0.5	0.021
10	7	10^5	3.5	0.1	150	0.5	1.0	0.034
11	4	10^5	1.5	0.1	150	0.5	0.5	0.025
12	7	10^5	1.5	0.1	200	1.0	1.0	0.01

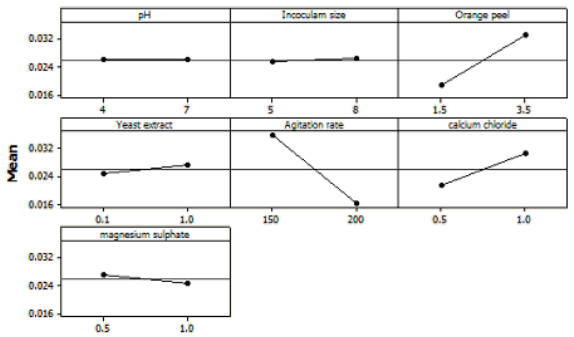


Fig 1. Main effect plots of the variables selected in PBD

Table 4: Central Composite design (CCD) experimental results of real values

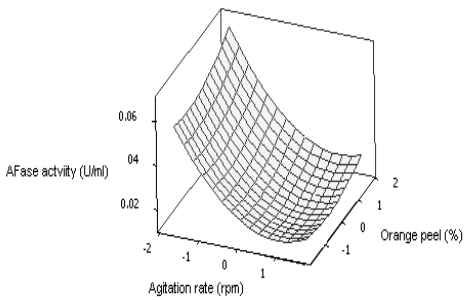
Exp.Run	*Orange peel (%)*	*Agitation rate (rpm)*	*$CaCl_2$ (g/l)*	*Enzyme activity (U/ml)*
1	2.5	132.96	0.75	0.052
2	2.5	175	0.75	0.017
3	1.5	150	0.5	0.027
4	1.5	200	1.0	0.016
5	2.5	175	0.75	0.015
6	2.5	175	1.17	0.024
7	2.5	175	0.75	0.019
8	3.5	200	1.0	0.019
9	3.5	150	0.5	0.036
10	3.5	150	1.0	0.041
11	2.5	175	0.33	0.016
12	2.5	217.05	0.75	0.01
13	2.5	175	0.75	0.016
14	4.18	175	0.75	0.027
15	2.5	175	0.75	0.014
16	1.5	150	1.0	0.039
17	3.5	200	0.5	0.017
18	0.82	175	0.75	0.011
19	1.5	200	0.5	0.014
20	2.5	175	0.75	0.012

Table 5: One way ANOVA for CCD experimental results

Source of variation	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F-value*	*P-value*
x_1	0.000381	4	0.000095	0.67	0.624
x_2	0.00216	4	0.00054	22.72	0.000
x_3	0.000304	4	0.000076	0.51	0.726

Table 6: CCD regression analysis results for full second-order polynomial equation

Source	*Coefficient*	*SE*	*t-Test*	*P*
Interception	0.01542	0.00131	11.782	0.000
$\mathbf{x_1}$	0.00322	0.00087	3.701	0.004
$\mathbf{x_2}$	-0.01081	0.00087	-12.445	0.000
$\mathbf{x_3}$	0.00252	0.00087	2.904	0.016
$\mathbf{x_1^2}$	0.00173	0.00085	2.041	0.068
$\mathbf{x_2^2}$	0.00597	0.00085	7.059	0.000
$\mathbf{x_3^2}$	0.00208	0.00085	2.46	0.034
$\mathbf{x_1\ x_2}$	-0.00063	0.00114	-0.551	0.594
$\mathbf{x_1\ x_3}$	-0.00063	0.00114	-1.432	0.183
$\mathbf{x_2\ x_3}$	-0.00088	0.00114	-0.771	0.459



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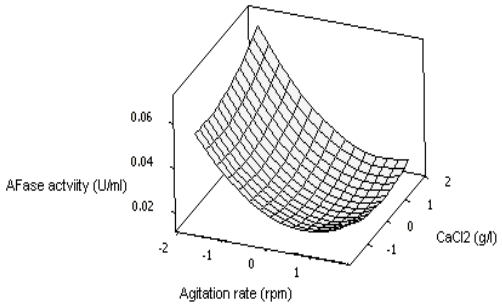

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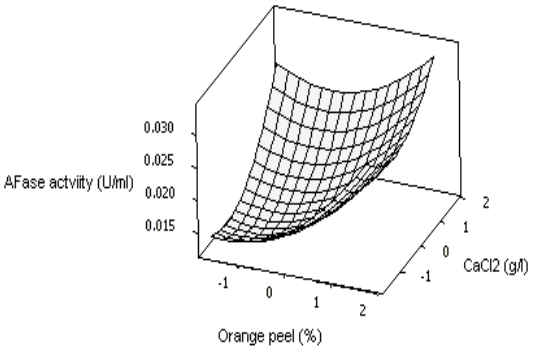

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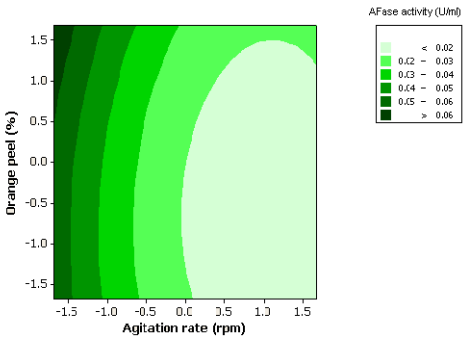

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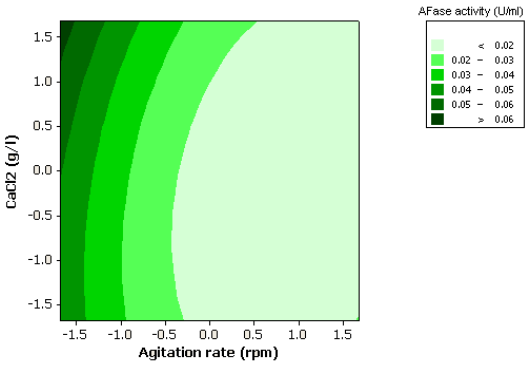

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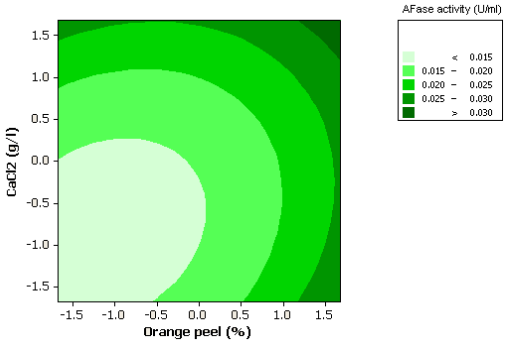

f)

Fig.2. Response surface and contour diagrams of AFase activity as a function of: (a, d) Orange peel and agitation rate, (b, e) $CaCl_2$ and agitation rate, (c, f) Orange peel and $CaCl_2$

RESULTS

Soil collected from the foot hills of Western Ghats (Anaikkatti) was screened for the presence of fungal species. The pure culture technique and the lactophenol cotton blue test revealed the presence of *Penicillium* species i.e., square shaped conidiophores of phialides. The broth culture filtrate analysis of *Pencillium* species has revealed about 0.004 U/ ml of α-L-arabinofuranosidase activity.

Plackett-Burman design

The PBD was used to investigate the effects of seven variables and submerged fermentation (SuF) technology was adopted for α-L-arabinofuranosidase production. The design matrix selected for screening the significant variables and their corresponding responses are given in Table 3. Among the variables screened, agitation rate (150 rpm), orange peel (3.5%) and $CaCl_2$ (1.0 g/l) were identified as the most significant variables influencing α-L-arabinofuranosidase production and the maximum activity was observed as 0.055 U/ml i.e., 10 fold increase in the activity.

Among the variables orange peel and $CaCl_2$ were found to exert a positive effect i.e., when the concentration was increased the AFase production was increased. In contrast, the agitation rate was observed to have a negative effect on α-L-arabinofuranosidase production i.e., when the rpm was increased the AFase production was decreased and these variables were found to be significant at 5% level ($p<0.05$). The other variables were found to be statistically insignificant at 5% and were, therefore, not considered in for further CCD analysis. The effect of all the variables on the activity of AFase was shown in Fig 1. The effect of the selected variables excluding orange peel and agitation rate has been previously reported for general hemicellulase enzymes [3].

Central Composite Design

CCD is a very useful tool for determining the optimal level of medium constituents and their interaction [17]. The levels of CCD were designed based upon the results obtained from PBD and SuF technology was adopted for α-L-arabinofuranosidase production. By keeping 10 day old inoculam as fixed, the optimum levels of the selected variables according to CCD were found to be 2.5% (orange peel), 132.95 rpm (agitation rate) and 0.75 g/l ($CaCl_2$) and the maximum activity was recorded as 0.052 U/ml at run No.1 (Table 4). From multiple regression analysis, it was found that the second-order polynomial equation can explain AFase production regardless of the significance of coefficients:

$$Y = 0.0998 + 0.00283\, x_1 - 0.000528\, x_2 + 0.0113\, x_3 + 0.00172627\, x_1^2 + 9.55 \times 10^{-6}\, x_2^2 + 0.03327\, x_3^2 + 2.5 \times 10^{-5}\, x_1 x_2 - 0.0035\, x_1 x_3 - 2.6 \times 10^{-4}\, x_2 x_3$$

where Y is the response value. In current experiment, Y value is the level of α-L-arabinofuranosidase production (U/ml). x_1, x_2 and x_3 represent the coded levels of orange peel, agitation rate and $CaCl_2$, respectively. The goodness of fit manifested by determination coefficient ($R^2 = 0.92$) showed that the sample variation of 92% for AFase production is characteristic to the independent variables and only 8% of the total variation cannot be explained by the model.

ANOVA was applied using the enzyme activity values were depicted in Table 5. ANOVA classifies and cross-classifies statistical results and tests whether the means of a specified classification differ significantly or not. This was carried out using Fisher's statistical test for the analysis of variance [18]. The analysis proved that agitation rate was the most controlling factor that affect the production of AFase (Statistically significant at 5% level, $p<0.05$) followed by orange peel and $CaCl_2$. A better R-value (85.83%) and an F-value higher than the listed value for 95% confidence was observed for agitation rate. The Student's-t-Test and its corresponding P-value also suggested that among the independent variables x_2 (agitation rate) possess significant effect in the production of Afase (Table 6).

The positive coefficients for orange peel (x_1) and $CaCl_2$ (x_3) revealed a linear effect in the increased production of AFase, whereas, the negative coefficient of agitation rate (x_2) indicated an inverse effect (i.e., increase in the rate leads to decrease production of AFase and vice versa). The cross-product results clearly showed that the most noteworthy interaction was occurred between agitation rate (x_2) and orange peel (x_1), and also equally between agitation rate and $CaCl_2$ (x_3). The response surface and contour plots were employed to determine the interaction between the variables and the optimum levels that have the most significant effect on AFase production (Fig 2). The surface and contour diagrams indicated that maximum AFase activity (> 0.05 U/ml) can be obtained by a combination of 2.5% (orange peel), 132.95 rpm (agitation rate) and 0.75 g/l ($CaCl_2$).

DISCUSSION

Obviously, it was proved that the experimental runs at centre points (run No.2, 5, 7, 13, 15 and 20) didn't record the maximum AFase activity and this could be because of the inverse action of agitation rate. Agitation is very much essential for proper diffusion and access of oxygen and all substrates for growing cells [19]. A report for xylanase documented in [20] has proved that a decrease in agitation rate has increased the enzyme production from *Aspergillus carneus* M34. In our present investigation, the same result was observed i.e., high agitation rate (217.05 rpm) has drastically decreased the AFase activity (0.01 U/ml), whereas, a very low agitation rate of 132.96 rpm has shown an increased activity (0.052 U/ml), perhaps because the higher sheering force causes the cells to lyse and the release proteases may degrade the produced AFase. Even though oxygen is essential for the growth of aerobic cultures, high agitation rate may cause air dispersion problem and affect the cell biomass concentration [21] and thereby the production of AFase.

Waste materials from a wide range of agro-industrial processes may be used as the substrates for microbial growth, thereby resulting in upgrading of the waste or the synthesis of valuable by-products. Microorganisms are capable of utilizing the organic matter in wastes both as a source of energy for growth and as carbon source for the synthesis of cell biomass [22]. Several agro wastes like banana peel, wheat bran, sugar cane bagasse and orange bagasse act as a cheap, cost effective natural source for the production of various industrial important enzymes [23]. Orange peel is considered to have high concentration of cellulose, hemicellulose and pectin which can act as potent carbon source and have been used for the production of polygalactouranase and pectin methyl esterase [24]. Previous scientific documentation have revealed that the fungal species belonging to the genera *Trichoderma*, *Aspergillus*, *Penicillium*, *Chaetomium*, *Fusarium* and *Piptoporus* were prominent producers of cellulases, hemicellulases and amylases [25]. To our knowledge, it is the first report on α-L-arabinofuranosidase production from *Penicillium* species using orange peel as a sole carbon source and it was found that 2.5% orange peel was the optimum concentration for AFase production.

Among different metal salts analyzed, previous reports have revealed that Ca^{2+} ions play a significant role in the stimulation of α-L-arabinofuranosidase activity to an appreciable level [26, 27]. This stimulatory activity was probably because of binding of Ca^{2+} ion in the non catalytic region may involve in the conformational change of the substrate binding site that perhaps cause an efficient hydrolysis. Our current investigation on the role of calcium ions supplemented as $CaCl_2$ also supported the stimulatory activity of AFase and 0.75 g/l was found to be the optimum concentration.

CONCLUSION

Central composite design a type of response surface analysis that was considered to be an effective tool was used to optimize the α-L-arabinofuranosidase production through SuF technology. The PBD experimental analysis has revealed that three variables orange peel (x_1), agitation rate (x_2) and $CaCl_2$ (x_3) were found to be significant in producing AFase. The CCD experimental analysis has predicted the α-L-arabinofuranosidase activity as 0.052 U/ml under the optimal media conditions as: orange peel (x_1) = 2.5%, agitation rate (x_2) = 132.95 rpm and $CaCl_2$ (x_3) = 0.75 g/l.

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